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Original Article

Ultrasonic-induced tonic convulsion in rats after subchronic exposure to perfluorooctane sulfonate (PFOS)

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ABSTRACT — Perfluorooctane sulfonate (PFOS) is one of the persistent organic pollutants distributed widely in the global environment. We have found that a single oral administration of PFOS induced tonic convulsion in mice and rats when a brief ultrasonic stimulus was applied to the animals. The aim of this study is to examine whether the neurotoxicity is caused by subchronic dietary exposure to PFOS. Rats were treated with dietary PFOS at 0, 2, 8, 32 and 128 ppm for 13 weeks. Animals were carefully observed for pharmacotoxic signs and responses to the ultrasonic stimulus applied biweekly. PFOS increased liver weight and decreased food consumption and body weight. PFOS concentrations in the serum, brain, liver and kidney were increased almost proportional to its total dose, although the ratios of PFOS concentrations in tissues to total doses in the group treated with the highest concentration were a little lower. The ranges of relative concentrations in the brain, liver and kidney to serum concentration were 0.13 to 0.24, 2.7 to 6.3 and 0.82 to 1.6, respectively. PFOS alone did not cause any neurotoxic symptoms; however, 5 rats out of 6 showed tonic convulsion in the 6th week when ultrasonic stimulus was applied to the 128 ppm rats with the total PFOS dose of 338 mg/kg. The ultrasonic stimulus did not cause convulsion in the other groups. Histopathological examination including electron microscopic examination could not detect any abnormality in the brain. Because the acute oral dose of PFOS causing the convulsion was 250 mg/kg (Sato *et al.*, 2009), the convulsion induced by PFOS seemed to depend on its total dose regardless of treatment schedule.

Key words: PFOS, Neurotoxicity, Convulsion, Subchronic

INTRODUCTION

Perfluorinated compounds (PFCs) have been used as refrigerants, surfactants, polymers, and as components of fire retardants, lubricants, adhesives, paper coatings, pharmaceuticals, cosmetics and insecticides. A major PFC, perfluorooctane sulfonate (PFOS), is regarded as a persistent organic pollutant that is distributed over the global environment. Many investigations revealed the environmental level of PFOS in rivers, lakes, seas and tap water (Saito *et al.*, 2004; Hansen *et al.*, 2002; Lange *et al.*, 2007; Jin *et al.*, 2009). Typically, the levels of PFOS in surface water are in the ppt range (Saito *et al.*, 2004). PFOS concentrations in a wide variety of wild animals are at ppb to ppm levels, which are 1,000 times higher than their environmental level (Giesy and Kannan, 2001; Martin *et al.*, 2004), indicating the bioaccumulative characteristics of this chemical. Almost the same levels of PFOS as in wild animals are also detected in human sera and milk (Olsen *et al.*, 1999; Kärrman *et al.*, 2007).

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Various harmful effects of PFOS have been reported in recent years, for example, increased liver weight, degeneration of hepatocytes, increased serum ALT activity, and decreased serum cholesterols and triglyceride (Seacat *et al.*, 2002, 2003). Reproductive toxicities such as malformation, and decreased neonatal body weight and survival rate have also been reported (Lau *et al.*, 2003, 2004; Thibodeaux *et al.*, 2003, Yahia *et al.*, 2008). Genotoxicity has not been demonstrated, but PFOS is suspected to cause bladder cancer (Alexander *et al.*, 2003). There are few reports on the neurotoxicity of PFOS. We found that a high dose of PFOS has a convulsive effect on mice and rats, and this convulsion is induced by brief exposure to ultrasonic stimulus (Sato *et al.*, 2009). As PFOS has non-degradative and bioaccumulative properties (Key *et al.*, 1997, 1998; Giesy and Kannan, 2001; Martin *et al.*, 2004), it is considered to be important to examine whether the same type of neurotoxicity is caused by its long-term exposure at lower levels. Therefore, we employed ultrasonic stimulus to detect the neurotoxicity of PFOS during 13 weeks of dietary exposure to 0 to 128 ppm PFOS in addition to the conventional Irwin method (Irwin, 1967).

MATERIALS AND METHODS

Chemicals and animals

PFOS potassium salt was purchased from Fluka Chemie GmbH (Buchs, Switzerland). Most of the other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Male Wistar rats were obtained from Clea Japan Inc. (Tokyo, Japan) at 4 weeks of age. They were fed commercial powder diet MF (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum* during the acclimatization period of one week. Animals were kept at 23 ± 1°C under a 12 hr light-dark cycle. This experiment was approved by the Animal Research Committee, Faculty of Agriculture, Iwate University, and was conducted according to the Guidelines for Animal Experiments of Iwate University.

PFOS-added diets were prepared by mixing the powder diet with small amounts of aqueous solution of PFOS to make concentrations of 0, 2, 8, 32 and 128 ppm. These diets were stored in a refrigerator until use.

Treatment and observations

Rats randomly divided into 5 groups were housed in a cage for each dose and given the PFOS-added diet (0, 2, 8 and 32 ppm: n = 5; 128 ppm: n = 6) and tap water *ad libitum* during the experimental period of 13 weeks. Food consumption in each cage was measured every day and divided by the number of rats in the cage to obtain the individual value. Body weight and rectum temperature were measured weekly and biweekly, respectively. Daily PFOS dose and total dose were calculated as follows:

Daily PFOS dose (mg/kg/day) = (PFOS concentration in food) × (weekly food consumption) / (mean body weight at that time) / 7.

Total dose (mg/kg) = (PFOS concentration in food) × (total food consumption by that time) / (mean body weight at that time).

The animals were carefully observed for pharmacotoxic signs, especially for excitability, such as Straub tail, tremors, twitches, convulsions, restlessness, alertness and motor activities. Responses, such as startle response, touch response, pain response, righting reflex, visual placing, abdominal tone and limb tone were examined according to the method of Irwin (1967). Responses of rats to the ultrasonic stimulus of 47 kHz for 10 sec at 30 cm from an ultrasonic machine (B42-JK, Branson Ultrasonics Corporation, Danbury, CT, USA) were observed biweekly.

Sampling

After 13 weeks of exposure, rats were deeply anesthetized with diethyl ether to collect blood from the vena cava. Serum samples were isolated and stored in a freezer at -18°C until use. Immediately after the blood collection, the animals were euthanized by bleeding to obtain the brain, liver and kidney, and their weights were measured. Brain samples were cut lengthwise into 2 portions. The halved brain, liver and kidney were stored in a freezer at -18°C for determination of PFOS. The remaining portions of the brain were fixed in neutral buffered 10% formalin for histopathological examination. For electron microscopic examination, small portions of the brain tissue were cut into approximately 2 to 3 mm^3 and fixed in 2.5% glutaraldehyde solutions overnight at 4°C.

Histopathological examination

Fixed brain samples were embedded in paraffin wax, cut into 4 μm sections and stained with hematoxylin-eosin (HE) for histopathological examination. Serial sections of the brain were incubated with Luxol fast blue, and incubated with neurofilament protein (DAKO Japan, Kyoto, Japan) and glial fibrillary acidic protein (DAKO Japan) antibodies to detect any damage to neuronal or glial cells. After overnight incubation, the sections were applied for the immunohistochemical detection system designed for rat tissue (Histofine Simple Stain Rat MAX-PO, Nichirei, Tokyo, Japan) and visualized using DAB chromogen.

Electron microscopic examination

The brain tissues were postfixed in 1% osmic acid for 2 hr, routinely processed and embedded in epoxy resin. The embedded blocks were cut into 50 to 70 nm, and the ultrathin sections were stained with double staining of uranyl acetate and lead nitrate (Nissin EM, Tokyo, Japan). After checking suitability of the brain tissues using semithin section stained with toluidine blue, the neurons in the cortex, hippocampus or cerebellum were examined using an electron microscope (JEOL 100S, Tokyo, Japan).

Determination of PFOS

The tissues were homogenized with 4 volumes of ultra pure water using a coaxial high-speed homogenizer. One milliliter of these homogenates and sera were mixed with 1 ml of 0.5 M tetrabutylammonium hydrogensulfate (pH 10) and 2 ml of 0.25 M sodium carbonate, and then stirred well. Five milliliters of methyl tertiary-butyl ether (MTBE) was added to the mixture, shaken for 1 min and centrifuged for 10 min at 3,000 rpm. Aqueous layer was removed and treated with MTBE once more. First and second MTBE fractions were combined, dried with nitrogen gas, dissolved in 1 ml of 90% methanol using an ultrasonic machine and filtered with 0.2 μm nylon filter (Autovial, Whatman Japan K.K., Tokyo, Japan). The filtrates were analyzed by LC/MS (Agilent 1100 LC/MSD, Agilent Technologies, Santa Clara, CA, USA) to determine the concentration of PFOS in tissues. Conditions of LC/MS are shown in our previous report (Sato *et al.*, 2009).

Statistical analysis

Fisher's exact test was used to compare the incidence of neurotoxic symptoms. Sign test with Bonferroni's correction was used to compare the food consumption of each group. Body weight and organ weight were compared by Dunnett's test or Welch's t-test with Bonferroni's correction for homoscedastic data and heteroscedastic data, respectively. Correlation coefficient and its significance between total dose of PFOS and its concentration in each tissue at the end of the subchronic study were calculated by Pearson's method. A p-value less than 0.05 was considered statistically significant.

RESULTS

No behavioral abnormalities that suggest a neurotoxic effect of PFOS were detected by the careful observation during the experimental period (data not shown). Biweekly ultrasonic stimulus caused no distinctive effects except for startle response in the rats other than the 128 ppm group, in which 5 rats out of 6 showed tonic convulsion at 6 weeks when the total ingested PFOS was 338 mg/kg (Table 1). The convulsion lasted several tens of seconds. All of the animals recovered from the convulsion but one of them was found dead the next morning. Therefore, the ultrasonic stimulus was discontinued for rats in the 128 ppm group to enable them to survive until the end of the experiment.

Food consumption was decreased in the 32 and 128 ppm PFOS groups (Table 1). After 2 weeks of exposure, body weight also decreased significantly at 32 and 128 ppm PFOS levels. Absolute and relative liver weights in the 128 ppm group and relative liver weight in the 32 ppm group were significantly increased (Table 2). Relative brain weight was significantly increased in the 32 and 128 ppm groups without change in the absolute weight. There were no remarkable changes in rectum temperature (data not shown).

At the termination of the experimental period, the highest and lowest concentrations of PFOS were found in the liver and brain, respectively. The ranges of relative concentrations in brain, liver and kidney to serum concentration were 0.13 to 0.24, 2.7 to 6.3 and 0.82 to 1.6, respectively (Table 3).

PFOS concentrations in the serum, brain, liver and kidney were increased almost proportional to its total dose, although the ratios of PFOS concentrations in tissues to total doses for the group with the highest concentration were a little lower (Table 4). Correlation coefficients between total dose and PFOS concentrations in the serum, brain, liver and kidney were highly significant (0.95, 0.93, 0.95 and 0.97, respectively, $p < 0.01$). Regression lines were $y = 0.62x + 37$ in serum and $y = 0.16x + 0.63$ in brain. PFOS concentrations in serum and brain at 6 weeks when ultrasonic-induced tonic convulsion was observed were estimated to be 246 and 55 mg/kg, respectively, using the regression line and total dose at that time.

In a previous study, 125, 250 and 500 mg/kg PFOS was administered to 8-week-old male Wistar rats by single oral intubation. Then, the PFOS concentration was measured at 24 hr after the treatment (Sato *et al.*, 2009). Comparing the previous acute and present subchronic studies, the PFOS concentrations of the tissues were very similar at the same total dose (Fig. 1).

Histopathologically, no changes were detected in the neuronal or glial cells of the cerebrum and cerebellum stained with HE, Luxol fast blue and neurofilament protein and GFAP in the treated groups (data not shown). In addition, no ultrastructural abnormalities of the neurons in the cortex and hippocampus, neurons and granu-

Table 1. Food consumption, body weight, PFOS dosage and number of convulsive rats

Week	0	1	2	3	4	5	6	7	8	9	10	11	12	13
0 ppm														
Food consumption (g/rat/week)		115	145	134	144	139	136	135	121	126	133	120	121	130
Mean body weight (g, n = 5)	96.4	152	197	246	287	307	322	339	346	364	379	392	400	416
S.D.	5.0	6.7	9.4	11.2	15.2	15.3	13.5	10.7	16.0	18.8	19.3	18.7	19.2	24.2
Convulsion		(-)	0/5	(-)	0/5	(-)	0/5	(-)	0/5	(-)	0/5	(-)	0/5	(-)
2 ppm														
Food consumption (g/rat/week)		117	117	137	135	119	154	138	130	127	131	122	110	113
Mean body weight (g, n = 5)	104	158	195	242	268	293	311	345	339	354	376	386	388	401
S.D.	2.9	6.3	10.0	12.1	10.3	12.5	10.4	12.0	11.9	13.8	14.8	14.4	11.8	17.8
Daily PFOS dose (mg/kg/day)		0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Total dose (mg/kg)		1.5	2.4	3.1	3.8	4.3	5.0	5.3	6.2	6.6	6.9	7.4	7.9	8.2
Convulsion		(-)	0/5	(-)	0/5	(-)	0/5	(-)	0/5	(-)	0/5	(-)	0/5	(-)
8 ppm														
Food consumption (g/rat/week)		109	109	135	132	127	135	127	118	129	129	121	116	122
Mean body weight (g, n = 5)	103	153	190	237	274	296	308	330	325	346	357	370	378	387
S.D.	4.7	9.9	12.5	13.2	5.9	14.1	14.0	13.3	14.0	13.3	10.7	12.1	14.4	14.3
Daily PFOS dose (mg/kg/day)		0.8	0.7	0.6	0.6	0.5	0.5	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Total dose (mg/kg)		5.7	9.2	11.9	14.1	16.5	19.4	21.2	24.4	25.9	28.0	29.6	31.5	33.3
Convulsion		(-)	0/5	(-)	0/5	(-)	0/5	(-)	0/5	(-)	0/5	(-)	0/5	(-)
32 ppm														
Food consumption (g/rat/week) *		113	108	124	127	130	123	129	108	122	120	116	131	126
Mean body weight (g, n = 5)	93.0	146	179*	223*	262*	279*	287**	305*	309**	327*	338**	347**	343**	357**
S.D.	5.4	8.1	9.0	10.1	14.5	10.7	11.2	12.5	12.5	15.4	140	10.1	10.6	11.8
Daily PFOS dose (mg/kg/day)		3.5	2.8	2.5	2.2	2.1	2.0	1.9	1.6	1.7	1.6	1.5	1.7	1.6
Total dose (mg/kg)		24.8	39.4	49.5	57.7	69.2	80.9	89.5	99.8	106	114	122	135	141
Convulsion		(-)	0/5	(-)	0/5	(-)	0/5	(-)	0/5	(-)	0/5	(-)	0/5	(-)
128 ppm														
Food consumption (g/rat/week) **		108	98.0	118	120	103	105	113	107	120	113	111	108	85.2
Mean body weight (g, n = 6 or 5)	91.7	143	169**	207**	233**	243**	248**	258**	257**	259**	285**	296**	296**	279**
S.D.	3.9	6.5	8.2	11.6	16.7	20.4	29.1	27.1	25.5	27.7	28.7	28.8	28.7	28.8
Daily PFOS dose (mg/kg/day)		13.9	10.6	10.4	9.4	7.8	7.8	8.0	7.6	8.5	7.2	6.8	6.7	5.6
Total dose (mg/kg)		97.3	156	200	244	288	338	380	435	490	496	526	574	647
Convulsion		(-)	0/6	(-)	0/6	(-)	5/6**	(-)	(-)	(-)	(-)	(-)	(-)	(-)

Convulsion (number of convulsive rats/number of rats in group). In 128 ppm group, numbers of rats were 6 and 5 for 0 to 6 weeks and 7 to 13 weeks, respectively. *$p < 0.05$, **$p < 0.01$: significantly different from the 0 ppm group. (-): not examined.

Table 2. Organ weights at the end of the study

		Brain	Liver	Kidney	Whole body
0 ppm	Absolute (g)	1.97 ± 0.06	16.1 ± 2.71	2.84 ± 0.68	416 ± 24.2
	Relative (%)	(0.47 ± 0.01)	(3.86 ± 0.55)	(0.68 ± 0.15)	
2 ppm	Absolute (g)	1.97 ± 0.05	14.4 ± 1.89	2.62 ± 0.58	401 ± 17.8
	Relative (%)	(0.49 ± 0.02)	(3.60 ± 0.48)	(0.65 ± 0.15)	
8 ppm	Absolute (g)	1.81 ± 0.08	14.6 ± 0.82	2.49 ± 0.24	387 ± 14.3
	Relative (%)	(0.47 ± 0.02)	(3.76 ± 0.13)	(0.64 ± 0.08)	
32 ppm	Absolute (g)	1.88 ± 0.05	18.2 ± 2.11	2.78 ± 0.71	357 ± 11.8 **
	Relative (%)	(0.53 ± 0.02) *	(5.10 ± 0.48) *	(0.78 ± 0.19)	
128 ppm	Absolute (g)	1.93 ± 0.07	20.8 ± 4.36 *	2.39 ± 0.53	279 ± 24.8 **
	Relative (%)	(0.70 ± 0.08) *	(7.42 ± 1.17) **	(0.86 ± 0.15)	

Relative = (organ weight)/(whole body weight) × 100. Mean ± S.D. n = 5. *p < 0.05, **p < 0.01, significantly different from the 0 ppm group.

Table 3. PFOS concentration in tissues at the end of the study

	PFOS concentration (mg/kg)			
	Serum	Brain	Liver	Kidney
2 ppm	9.50 ± 0.68	1.91 ± 0.37	59.7 ± 8.96	14.8 ± 4.60
8 ppm	44.1 ± 5.60	6.91 ± 1.38	135 ± 42.7	36.0 ± 11.2
32 ppm	177 ± 20.0	22.3 ± 114	647 ± 113	188 ± 46.8
128 ppm	432 ± 75.3	105 ± 19.8	1180 ± 156	628 ± 169
	Relative concentration to serum			
	Serum	Brain	Liver	Kidney
2 ppm	1.0 ± 0.07	0.20 ± 0.04	6.3 ± 0.94	1.6 ± 0.48
8 ppm	1.0 ± 0.13	0.16 ± 0.03	3.1 ± 0.97	0.82 ± 0.26
32 ppm	1.0 ± 0.11	0.13 ± 0.06	3.7 ± 0.64	1.1 ± 0.26
128 ppm	1.0 ± 0.17	0.24 ± 0.05	2.7 ± 0.36	1.5 ± 0.39

Mean ± S.D. n = 5.

lar cells in the cerebellum were found in the PFOS-treated group (Fig. 2).

DISCUSSION

Seacat *et al.* (2003) fed 0.5, 2, 5 and 20 ppm PFOS in a diet to male Sprague Dawley (SD) rats for 14 weeks and observed an increase in liver weight and a decrease in the food consumption at 20 ppm, but no change in terminal body weight. In the present study, an increase in liver weight at 128 ppm and decreases in food consumption and body weight at 32 and 128 ppm were observed. The mechanisms of the increase in liver weight and decrease in body weight have not been reported yet. However,

Table 4. Ratios of PFOS concentrations in tissues to total doses at the end of the study

	Total dose (mg/kg)	Concentration/Total dose			
		Serum	Brain	Liver	Kidney
2 ppm	8.20	1.2 ± 0.08	0.23 ± 0.05	7.3 ± 1.1	1.8 ± 0.56
8 ppm	33.3	1.3 ± 0.17	0.21 ± 0.04	4.0 ± 1.3	1.1 ± 0.34
32 ppm	141	1.3 ± 0.14	0.16 ± 0.08	4.6 ± 0.80	1.3 ± 0.33
128 ppm	647	0.67 ± 0.12	0.16 ± 0.03	1.8 ± 0.24	0.97 ± 0.26

Mean ± S.D. n = 5.

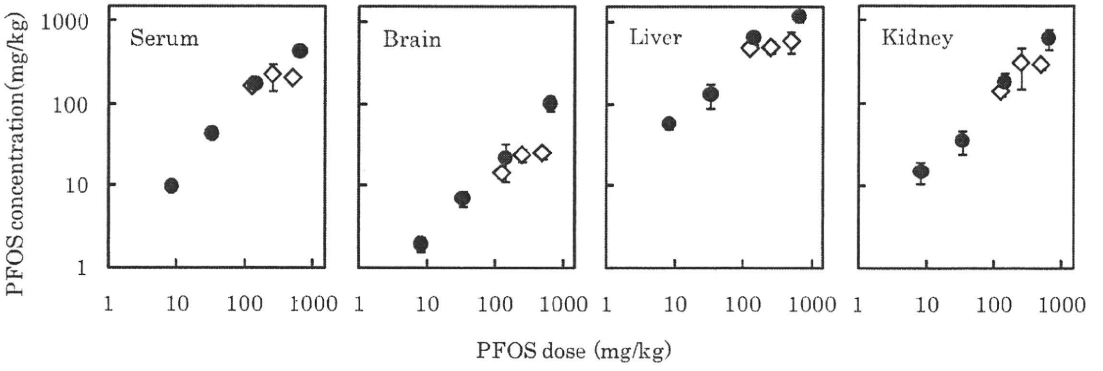

Fig. 1. Relationship between acute and subchronic exposure in PFOS concentration to total dose. ●: Subchronic exposure (present results), ◇ : acute exposure (cited from Sato *et al.*, 2009). Mean ± S.D. n = 5 (subchronic exposure), n = 3 (acute exposure).

the reason for the difference in the body weight change between the present study and that of Seacat *et al.* (2003) may be the difference in PFOS doses.

A radiolabeling study in which adult male rats were given a single intravenous dose demonstrated that the concentration of PFOS in the liver is higher than that in the plasma and kidney (3M Company, 2003). Our results showing that PFOS concentration was highest in the liver among the examined tissues support this report.

To our knowledge, there has been no report about PFOS concentration in the brain of subchronically treated animals. This study first determined PFOS concentration in brain treated subchronically with a wide range of PFOS, and revealed that the brain showed the lowest PFOS concentration among the tissues examined. The PFOS concentration in brain was about 5 times lower than that in serum. However, convulsion was observed in the 128 ppm group. The animals had ingested 338 mg/kg PFOS when the convulsion was observed. In a single oral exposure study, the convulsion was caused by a dose of 250 mg/kg or more PFOS (Sato *et al.*, 2009). This suggests that the total amount of PFOS required for convulsion is almost the same regardless of treatment schedule. One of the reasons for this may be that PFOS concentration in the brain (as well as other organs tested) in the subchronic study was very similar to that of an acute study at the same total dose, as shown in Fig. 1. Johnson *et al.* (1979) intravenously administered ^{14}C-PFOS to male rats, and recovered 30.2 ± 1.5% and 12.6 ± 1.2% from urine and feces, respectively, by 89 days after administration. These elimination data suggest that the biological half-life of PFOS is 110 days in rats. This long half-life may be a reason for the similar concentrations of PFOS between acute and subchronic treatments.

In general, the increase of excitatory neurotransmitters or decrease of inhibitory ones is involved in the pathogenesis of convulsion. However, there were no changes in glutamic acid, glycine, GABA, norepinephrine, dopamine and serotonin concentrations in rat brain treated with 250 mg-PFOS/kg p.o., which was enough to cause convulsion

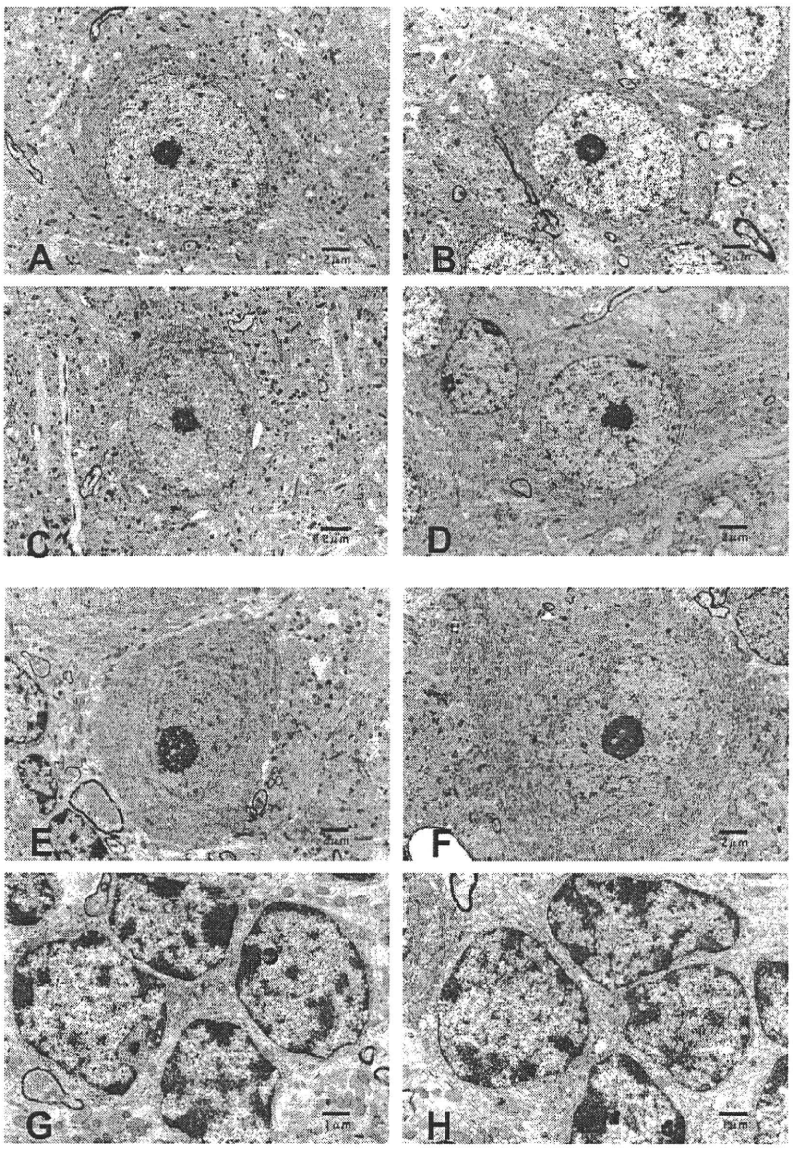

Fig. 2. Ultrastructures of neurons in the cortex (A,B) and hippocampus (C, D), Purkinje cells from the cerebellum (E, F) and the granule cells in the granular layer of the cerebellum (G, H). A, C, E and G are obtained from the 0 ppm group, and B, D, F and H being from the 128 ppm group. Bar indicates each magnification. No abnormalities in the neural cells related to treatment were observed.

(Sato *et al.*, 2009). In the present study, no morphological changes were also detected by not only light microscopic but also electron microscopic examinations. Thus, quantitative alteration of neurotransmitters and lesion of nerve cells may not be involved in PFOS-induced convulsion. On the other hand, PFOS increases Ca^{2+} influx by depolarizing membrane potential in paramecia (Kawamoto *et al.*, 2008), and affects the action potential and currents in rat cerebellar Purkinje cells (Harada *et al.*, 2006). As Ca^{2+} plays an important role in the tonic convulsion (Akaike and Himori, 2002) alteration of Ca^{2+} kinetics in nerve cell may be involved in the convulsive effect of PFOS.

In the present study, PFOS concentration in serum was estimated to be 246 ppm when ultrasonic-induced tonic convulsion was observed. This serum PFOS concentration was about 10,000 times higher than that in human or wild animals, the ranges of which are tens to hundreds of ppb (Giesy and Kannan, 2001; Olsen *et al.*, 2004). Higher serum PFOS concentration in fluorochemical production workers was reported to be 12.8 ppm (Olsen *et al.*, 1999), which is close to the present serum PFOS concentration. In addition, the convulsive effect of PFOS was detected only by the ultrasonic stimulus but not by the conventional Irwin method. Therefore, this unique neurotoxicity of PFOS should be examined further.

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